Identification of Enterotoxigenic *Escherichia coli* with Synthetic Alkaline Phosphatase-Conjugated Oligonucleotide DNA Probes

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Alkaline phosphatase-conjugated (AP) 26-base oligonucleotide DNA probes were compared with the same probes labeled with γ -³²P for the identification of heat-labile (LT) and heat-stable (ST) enterotoxigenic *Escherichia coli* (ETEC). The AP oligonucleotide probes were as sensitive as the radiolabeled (RL) probes in detecting LT and STA-2 target cell DNA, but the AP ST probe, which differed from STA-1 by two bases, was less sensitive than the RL probe in detecting STA-1 DNA (6.25 versus 0.78 ng). Of 94 ETEC that were identified with the RL probe, the AP probes detected 93% (28 of 30) of ST, 73% (25 of 34) of LT, and 67% (20 of 30) of LTST ETEC. When colony lysates of these ETEC were examined, the AP probes identified all 94 ETEC. In examinations of stool blots, the RL and AP probes were shown to have sensitivities of 71 and 59%, specificities of 91 and 86%, positive predictive values of 87 and 73%, and negative predictive values of 86 and 74%, respectively. AP oligonucleotide probes to detect ETEC were less sensitive in detecting ETEC by colony or stool blot hybridization than the RL probes but could be used by laboratories without access to radioisotopes to examine colony lysates.

Enterotoxigenic *Escherichia coli* (ETEC) is a frequent cause of diarrhea in children and nonimmune adults in tropical developing countries (2, 3, 13). ETEC is usually identified by testing isolates for enterotoxin production in bioassays or serological tests that detect phenotypic expression of the genes coding for heat-labile and heat-stable enterotoxins (LT and ST, respectively) (6, 10, 17, 21, 23). Alternatively, ETEC can be identified by detecting genes coding for these enterotoxins by DNA hybridization (7, 8, 14, 18); this method has been used when it is necessary to test large numbers of specimens. The disadvantage of DNA hybridization assays for routine diagnostic use or use in developing countries is the need for radioisotopes. Radioisotopes have a short half-life, are difficult to handle, and need to be shipped frozen from commercial sources.

Enteroinvasive *E. coli* and *Shigella* spp. can be identified with a nonradioactive biotinylated DNA probe (19). Biotin can be successfully used as a nonradioactive marker in a relatively large DNA probe (17 kilobases) because enough biotinylated nucleotides can be incorporated by nick translation. Biotin could also be used in a 850-base-pair DNA probe for genes coding for *E. coli* LT, but was not a sensitive marker for smaller DNA probes used to detect genes coding for ST because not enough molecules of biotin could be incorporated into these probes by nick translation. Oligonucleotide probes for ETEC, attached to a single molecule of biotin, lacked sensitivity in colony hybridization assays (8).

Synthetic oligonucleotide probes constructed from known DNA sequences of the genes coding for *E. coli* enterotoxins are more uniform and easier to use than the cloned enterotoxin gene probes (9). The sequences chosen for these synthetic oligonucleotides were taken from the DNA sequences of the LT and STA-2 genes (5, 12, 15, 22). The ST oligonucleotide sequence is homologous with STA-2 DNA

sequence and differs from the STA-1 sequence by two bases. Both the LT and ST probes are 26 bases long and were synthesized in a DNA synthesizer by using phosphoramidite chemistry (4).

Renz and Kurz constructed a nonradioactive DNA probe by attaching the enzyme alkaline phosphatase directly onto a DNA probe (16). Nonradioactive oligonucleotide probes were constructed by covalently linking alkaline phosphatase directly to the C-5 position of a thymidine base through a 12-atom spacer arm (11). These nonradioactive probes were compared with the same probes labeled with γ -³²P in identifying ETEC colonies and ETEC infections by probing bacterial growth of stools from children with diarrhea.

MATERIALS AND METHODS

Preparation of filters inoculated with purified DNA. Plasmid DNA coding for LT, STA-1, and STA-2 was isolated from *E. coli* C600(pEWD299), *E. coli* C600(pRIT10036), and *E. coli* C600(pSLM004), respectively, by ethidium bromide-cesium chloride centrifugation (18, 20). Decreasing concentrations of DNA, diluted in TE buffer, were spotted on nitrocellulose (NC) filters, baked at 80°C for 2 h in a vacuum oven, and examined with either the γ -³²P-labeled (RL) or alkaline phosphatase-conjugated (AP) oligonucleotide probe.

Preparation of filters inoculated with ETEC. ETEC isolated in Thailand, identified by the Y-1 adrenal and suckling mouse assays and examined for hybridization with the LT, STA-1, and STA-2 enterotoxin gene probes, were spotted on NC filters (BA 85; Schleicher & Schuell, Keene, N.H.), layered on MacConkey agar, and incubated at 37°C for 12 h (6, 8, 17). Filters were treated with 0.5 N NaOH and 1.0 M ammonium acetate-0.02 M NaOH, and the DNA of the bacterial colonies was fixed on NC filters by heating at 80°C for 2 h in a vacuum oven.

Preparation of filters inoculated with stool specimens. Stool samples collected from children with diarrhea were cultured

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FIG. 1. Detection of decreasing concentrations of pEWD299 (LT), pSLM004 (STA-2), and pRIT10036 (STA-1) spotted on NC paper. (A) Hybridized with 10^5 cpm of RL probes for 16 h and exposed to X-Omat X-ray film for 16 h at -70°C . (B) Hybridized with AP probes for 30 min and exposed to substrate for 4 h. Concentration of DNA: 1a, 50 ng; 1b, 25 ng; 1c, 12.5 ng; 1d, 6.25 ng; 2a, 3.12 ng; 2b, 1.56 ng; 2c, 0.78 ng; 2d, 0.39 ng; 3a, 0.19 ng; 3b, 0.095 ng; 3c, 0.047 ng; 3d, 0.023 ng.

on MacConkey medium at 37°C overnight. Ten *E. coli* isolates were saved on nutrient agar slants and tested for LT and ST in the Y-1 adrenal and suckling mouse assays (bioassays) (6, 17). The same stool samples were also inoculated on different pieces of NC paper, layered on MacConkey medium, and incubated at 37°C for 16 h. Filters were processed as described above and are referred to as stool blots.

Preparation of RL oligonucleotide probes. The 26-base LT and ST oligonucleotide probes were synthesized by using phosphoramidite chemistry (Applied Biosystems, Foster City, Calif.) (4). Fifty picomoles of each oligonucleotide probe was 5'-end labeled with $\gamma\text{-}^{32}\text{P}$ with T4 polynucleotide kinase as specified by the manufacturer (New England Nuclear Corp., Boston, Mass.).

Preparation of AP oligonucleotide probes. The enzyme alkaline phosphatase was covalently linked directly to the C-5 position of a thymidine base through a 12-atom spacer arm as described previously (11).

Hybridization with the RL oligonucleotide probe. Hybridization of filters with $\gamma\text{-}^{32}\text{P}$ -labeled probes was performed at 50°C for 30 min in a solution containing $6\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 1% bovine serum albumin, 1% polyvinylpyrrolidone, and 1 mM EDTA with $5\text{ }\mu\text{g}$ (10^6 cpm/ml) of oligomer probe per ml.

Hybridization with AP. For hybridization with AP probes, filters were placed in $1\times$ SSC with 2 mg of proteinase K (Bethesda Research Laboratories, Gaithersburg, Md.) per ml at 50°C for 15 min and then washed in $1\times$ SSC at 25°C . Filters were prehybridized in hybridization buffer ($6.25\times$ SSC, 0.5% bovine serum albumin, 0.5% polyvinylpyrrolidone, and 1% sodium dodecyl sulfate) for 30 min at 50°C . Filters were hybridized with 50 ng of AP probes per ml for 30 min at 50°C . This amount of probe was sufficient to hybridize 100 cm^2 of NC filter. Filters were then washed twice for 5 min each in $1\times$ SSC-1% sodium dodecyl sulfate.

Washed filters were then immersed in 7.5 ml of a solution containing 0.1 M Tris hydrochloride, 0.1 M NaCl, 0.05 M MgCl_2 , 0.1 M ZnCl_2 , and 0.02% sodium azide, pH 8.5, with $33\text{ }\mu\text{l}$ of 70% diethylformamide and $25\text{ }\mu\text{l}$ of 5-bromo-4-

TABLE 1. Identification of 94 ETEC by DNA hybridization with RL and AP probes

ETEC	No. of isolates identified		
	Bioassay	RL probes	AP probes
LTST	30 (4 STA-1, 26 STA-2)	30	20
LT	34	34	25
ST	30 (2 STA-1, 28 STA-2)	30	28

chloro-3-indolyl phosphate (50 mg/ml in diethyl formamide) and incubated for 16 h.

RESULTS

Detection of purified DNA by RL and AP probes. Both the AP and RL LT probes detected 1.56 ng of cloned LT plasmid DNA, and the AP and RL ST probes detected 0.39 ng of cloned STA-2 plasmid DNA (Fig. 1). The AP ST probe detected 6.25 ng and the RL ST probe detected 0.78 ng of cloned STA-1 plasmid DNA.

Examination of strains by colony hybridization with RL and AP probes. The results of examining ETEC by colony hybridization with the RL probes are shown in Table 1 and Fig. 2. Perfect correlation was observed between the results obtained with bioassays and with the RL-probed colonies. The AP probes detected 93% (28 of 30) of ST, 73% (25 of 34) of LT, and 67% (20 of 30) of LTST ETEC. Five LTST ETEC were not detected with the LT AP probe, and five other isolates were not detected with the ST AP probe. Both STA-1 and one of four LT STA-1 ETEC were not detected with the AP ST probe. Colony lysates of all ETEC were detected with the AP probes. None of 60 non-ETEC hybridized with the RL or AP probes by colony hybridization.

Detection of ETEC infections by examining stool blots with RL probes. ETEC were identified in 30 of 231 (13%) children with diarrhea by testing 10 *E. coli* isolates from each specimen in the bioassays and examining stool blots with the RL probes. ETEC infections were identified in 27 children by the bioassays and in 22 by examining stool blots with the RL probes (Table 2). The RL probes hybridized with stool blots from three children for whom ETEC were not identified with the bioassays, and stool blots from seven children with ETEC infections identified by the bioassay did not hybridize with the RL probes. Stool blots from two children with LTST ETEC infections, as identified in the bioassays,

FIG. 2. Hybridization of ETEC with AP ST (A) and RL ST (B) probes. Dots: 1a, D47 LT⁺ STA-2⁺ *E. coli*; 1b, CH132 LT⁺ STA-2⁺ *E. coli*; 1c, D83 LT⁺ STA-2⁺ *E. coli*; 1d, D110 LT⁺ STA-2⁺ *E. coli*; 2a, D114 LT⁺ ST⁻ *E. coli*; 2b, HV200 LT⁺ STA-2⁺ *E. coli*; 2c, HV170 LT⁺ ST⁻ *E. coli*; 2d, HV97 LT⁺ STA-2⁺ *E. coli*; 3a, D251 LT⁻ STA-1⁺ *E. coli*; 3b, D245, LT⁻ STA-1⁺ *E. coli*; 4b, B2C LT⁺ STA-1⁺ STA-2⁺ *E. coli*; 4c, LT⁻ ST⁻ *E. coli* K-12.

TABLE 2. Identification of 30 ETEC infections in children with diarrhea^a

ETEC	No. of infectious identified	
	Bioassay	RL probes
LTST	7	4
LT	12	10
ST	8	8

^a *E. coli* was tested for enterotoxin production in bioassays, and stool blots were examined for hybridization with the RL probes.

hybridized with only the LT or the ST RL probe. None of the stool blots collected from 30 children for whom ETEC were not identified with the bioassay hybridized with the RL probes.

Detection of ETEC infections by examining stool blots with the AP probes. Stool blots from 30 children infected with ETEC as identified in bioassays or with the RL probes and 31 children with diarrhea for whom ETEC infections were not identified by either method were examined with AP probes (Fig. 3). The RL probes were 71% sensitive and 91% specific and had a positive predictive value of 87% and a negative predictive value of 86%. The AP probes had a sensitivity of 59%, a specificity of 86%, a positive predictive value of 73%, and a negative predictive value of 74%. Both the RL and AP probes were more sensitive in detecting LT only and ST only than LTST ETEC infections (Table 3).

DISCUSSION

Practical methods of identifying ETEC are needed, especially in developing countries where ETEC are a frequent but often undetected cause of diarrhea. Most of the assays used to identify ETEC require tissue cultures, specific antisera, or animals (6, 10, 17, 21, 23). The GM-1 enzyme-linked immunosorbent assay and the Biken test have been proposed as practical methods of identifying LT ETEC (10, 23), but the standard test for ST ETEC is still the suckling mouse assay (6). Few laboratories, especially in the tropics, are able to maintain animal colonies large enough to test a large number of *E. coli* isolates. An indirect enzyme immunoassay to detect ST has been developed but depends on specific antisera (21). The development of a simple in vitro assay that could replace the suckling mouse assay was a research priority of the World Health Organization in 1986.

Radiolabeled cloned polynucleotide and synthetic oligonucleotide enterotoxin gene probes have been successfully used to identify ETEC in large numbers of specimens (7-9, 14, 18), but nonradioactive DNA probes had to be developed before this technique could have universal applications. Nonradioactive DNA probes that have been developed to detect enteropathogenic *E. coli* are not as sensitive as the radiolabeled versions (8, 19). The AP probes described in this report were as sensitive in detecting LT and STA-2 plasmid DNA as the RL probes, but the AP ST probe was less sensitive than the RL ST probe in detecting DNA sequences coding for STA-1, which differs from the ST probe by two base changes. The AP probes detected 93% of ST, 73% of LT, and 67% of LTST ETEC by colony hybridization. The failure of AP probes to detect 22% of ETEC by colony hybridization was presumably due to the difficulty of the probes in consistently reaching target cell DNA due to their being covalently linked to alkaline phosphatase. When bacterial lysates were examined, the AP probes identified all ETEC that were identified with the RL probes.

FIG. 3. Detection of ETEC infections by examining stool blots for hybridization with AP probes. (a and b) Stool blots from patient EI 1066, in whom LT⁻ ST⁺ ETEC was identified by bioassays, hybridized with the LT (a) and ST (b) AP probes; (c and d) stool blots from patient EI 1065, in whom LT⁺ ST⁻ ETEC was identified by bioassays, hybridized with the LT (c) and ST (d) AP probes; (e and f) stool blots from patient EI 983, in whom LT⁺ ST⁺ ETEC was identified by bioassays, hybridized with the LT (e) and ST (f) AP probes. LT⁻ ST⁻ *E. coli* K-12 was spotted on the lower left-hand corner, and LT⁺ ST⁺ *E. coli* B2C was spotted on the lower right-hand corner of each stool blot. In panels e and f the colored precipitates had fallen off the NC filters before this picture was prepared.

Enterotoxin gene probes have been used to examine *E. coli* isolates or the total bacterial growth from stool specimens. While examining stool blots is faster and easier to perform than testing individual *E. coli*, examining stool blots with RL probes only detected 77 to 84% of ETEC infections, although stool blots will occasionally detect genes coding for enterotoxin in specimens in which ETEC isolates were not identified by the bioassays (8). The AP probes described in this report provide a method of identifying ETEC by DNA hybridization which is less sensitive than the RL probe, but may still have application. Since AP probes are stable at room temperature when lyophilized, they can be transported and stored without refrigeration. These probes will enable clinical laboratories to detect genes coding for enterotoxins in colony lysates without reliance on frequent shipments of

TABLE 3. Comparison of results obtained with the RL and AP probes in detecting ETEC infections by examining stool blots

Probe and ETEC	Sensitivity (%)	Specificity (%)	Predictive value (%)	
			Positive	Negative
RL probes				
LTST	57	97	80	91
LT	100	97	90	100
ST	87	94	78	97
All	71	91	87	86
AP probes				
LTST	43	97	75	88
LT	100	94	78	100
ST	75	94	75	94
All	59	86	73	74

radioactively labeled nucleotides. AP probes can also be used to rapidly detect genes coding for enterotoxin by colony or stool blot hybridizations, but these probes are not as sensitive as RL probes.

Nonradioactive DNA probes can be used in a variety of other hybridization assays to detect DNA sequences encoding *E. coli* toxins. For example, the AP ST probe was recently used in our laboratory to detect DNA sequences encoding ST in plasmids transferred to NC paper. DNA hybridization assays have previously been available to only a few research laboratories in developing nations. This rapid and convenient method of identifying genes coding for enterotoxins will be extremely valuable in investigating epidemics of diarrhea caused by ETEC and defining the prevalence of ETEC in different populations. Examining bacterial lysates with the ST AP probes is an alternative in vitro method of identifying ST ETEC.

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